Available online at www.sciencedirect.com
SCIENCE DIRECT®
Aquaculture
Aquaculture 255 (2006) 76–81

Microbial conditions and antimicrobial activity in cultures of two microalgae species, *Tetraselmis chuii* and *Chlorella minutissima,* and effect on bacterial load of enriched *Artemia* metanauplii

Pavlos Makridis *, Rita Alves Costa, Maria Teresa Dinis

Center for Marine Sciences, University of Algarve, 8000-118 Faro, Portugal

Abstract

The microbial conditions and antimicrobial activity were determined in batch cultures of two microalgae species, *Tetraselmis chuii* and *Chlorella minutissima*. The number of bacteria associated with the microalgae cultures showed an exponential growth 2, 10, and 16 days after inoculation, and they were higher in *T. chuii* in all three sampling points compared with *C. minutissima*. No presumptive *Vibrio* strains were observed in any of the samples, as measured by the growth on TCBS agar. A total of 17 and 30 bacterial strains were isolated from *C. minutissima* and *T. chuii*, respectively. A high percentage of Gram-positive strains was detected among the bacterial strains isolated, as Gram-positive strains constituted 82% (14/17) and 73% (22/30) of the total numbers of isolates in *C. minutissima* and *T. chuii*, respectively. The isolated bacteria were screened in vitro for inhibition against two pathogenic strains, and nine of the 34 strains tested (26%) showed inhibition in vitro against either *Photobacterium damselae*, *susp. piscicida* or *Vibrio anguillarum*. Incubation of enriched *Artemia* in cultures of the two microalgae for 30 min resulted in a significant decrease of the bacterial load in *Artemia* ($P<0.05$), and a significant decrease of the level of presumptive *Vibrio* in *Artemia* homogenates ($P<0.05$). The results of this study demonstrate a simple and practical approach to decrease the microbial load and at the same time reduce the percentage of *Vibrio* among the bacteria associated with enriched *Artemia*.
© 2005 Elsevier B.V. All rights reserved.

1. Introduction

Microalgae are commonly used in the rearing of marine fish larvae. They are either added directly to the water in the rearing tanks, when applying the "green water" technique (Reitan et al., 1997), or used as food for rotifers. Addition of microalgae often has a positive effect on the survival rates of fish larvae (Oie et al., 1997) and on the species-composition of the gut microbiota of the larvae by lowering the numbers of opportunistic bacteria (Salvesen et al., 1999). In rotifer cultures, use of microalgae leads to a decrease in numbers of bacteria associated with the rotifers, when compared with cultures fed yeast-based diets (Oie et al., 1994).

Artemia nauplii and metanauplii are commonly used as live food in the rearing of marine fish larvae. During the hatching and enrichment processes, the numbers of bacteria in *Artemia* increase exponentially (Austin and Allen, 1982; Ritar et al., 2004). The bacterial microbiota associated with enriched *Artemia* metanauplii may harbour bacteria deleterious to the fish larvae, as the proportion of *Vibrio* bacteria in *Artemia* metanauplii

* Corresponding author. Present address: Hellenic Center for Marine Research P.O. Box 2214, GR 71003, Heraklion, Crete, Greece. Tel.: +30 2810 346860x136; fax: +30 2810 241882.
E-mail address: makridis@her.hcmr.gr (P. Makridis).

constitutes more than 50% of the total count of aerobic heterotrophic culturable bacteria (Olsen et al., 2000; Ritar et al., 2004). During the early development of the larvae, there is in an exponential proliferation of bacteria in the fish gut, caused mainly by the bacteria associated with live food (Makridis et al., 2000a). High mortalities in intensive rearing of marine fish larvae could be partly explained by the increased number of opportunistic bacteria which proliferate in the fish gut during this critical period (Olafsen, 2001), so control of the bacterial communities present in the live food could lead to higher survival rates and a more predictable outcome for the hatcheries. In the culture of flatfish species, it is a common practice to use frozen and thawed enriched *Artemia* metanauplii, which are thus an easy prey for post-larvae that have settled to the bottom of the tank (Imsland et al., 2003). If the *Artemia* added to the tanks have a high bacterial load, it is probable that after thawing the bacteria associated with *Artemia* may proliferate and have deleterious effects on the fish.

A strategy to modify the microbiota of *Artemia* was suggested by Makridis et al. (2000b), which included short-term incubation of *Artemia* in high concentration suspensions of bacteria innocuous to the fish larvae. However, several other approaches have been suggested for the control of the bacteria present in the *Artemia*, such as use of probiotics in rotifer cultures, formaldehyde, and incubation of *Artemia* in microalgae cultures; an approach that has been tested in cultures of juvenile *Artemia* (Olsen et al., 2000; Gatesoupe, 2002; Vilamil et al., 2003). Antimicrobial activity of microalgae cultures has been shown in earlier studies (Duff and Bruce, 1966; Austin et al., 1992). This antimicrobial activity could be explained by the bacteria present in the microalgae cultures (Dopazo et al., 1988), or by antimicrobial substances produced by the microalgae cells (Marshall et al., 2005).

The aims of this study were fourfold: (i) Isolate representative bacterial strains of the culturable aerobic microbiota in cultures of two microalgae species, *Tetraselmis chuii* and *Chlorella minutissima*, and measure the total bacterial load in different phases of batch cultures, (ii) Determine some basic phenotypic characteristics of the bacterial strains isolated and their capacity to inhibit in vitro pathogenic bacterial strains, (iii) Evaluate the effect of short-term incubation of enriched *Artemia* metanauplii in microalgae cultures on the microbial load of Artemia and (iv) Measure the bacterial content in frozen and thawed *Artemia*, which have been previously incubated in microalgae, to simulate the conditions during feeding of flatfish larvae with frozen *Artemia*.

2. Materials and methods

Two microalgae species were used in this study, *T. chuii* and *C. minutissima*, which were kindly provided by Dr. Luis M. Lubián, Instituto de Ciencias Marinas de Andalucía, Spain and Dr. Pascal Divanach, Hellenic Center for Marine Research, Greece, respectively. The microalgae were cultivated in F/2 medium (Guillard and Ryther, 1962) in five-litre cylindrical containers. Seawater in the medium (salinity 33 ppt) was previously sterilized by chlorination overnight with NaOCl (0.5 ml l^{-1}) and then de-chlorination for 3–4 h with sodium thiosulfate pentahydrate (0.3 g l^{-1}). The cultures were kept under constant illumination and aeration at 20–22 °C. Samples were taken 2, 10, and 16 days after inoculation to isolate representative bacterial strains of the culturable, aerobic microbiota of the microalgae cultures. Fifty ml of each culture were shaken vigorously and then centrifuged for 10 min at 3200 ×*g* to remove the microalgae cells. The supernatants and their 10-fold dilutions were plated in Zobell's marine agar (Zobell, 1946) and thiosulfate citrate bile salt (TCBS) agar dishes (Difco Laboratories, Sparks, MD, USA). The dishes were incubated at 20 °C for 7–10 days, the numbers of colony-forming units (CFU) were counted, and representative colonies from all samples were selected, based on the different colony characteristics (colour, shape, size, and timing of appearance). These colonies were sub-cultured to purity and stored in tryptic soy broth added 2% NaCl (TSBS) and 30% glycerol at −80 °C. Several biochemical tests were run to obtain some basic characteristics of the strains isolated (Smibert and Krieg, 1994): Gram staining, fermentation, oxidase, catalase, growth in Na^+-deficient medium, growth in TCBS, and detection of arginine, lysine and ornithine decarboxylase and amylase activity.

The isolated bacteria were screened in vitro for inhibition against two pathogenic strains using a double-layer approach previously described by Dopazo et al. (1988). The pathogenic strains used were *Photobacterium damselae susp. piscicida*, CECT 5895, and *Vibrio anguillarum* CECT 522 provided by the Collectión Espanõla de Cultivos Tipo (CECT). The identity of the pathogenic strains was verified by use of API-20E identification kit (Biomerieux Inc., Marcy l'Etoile, France). Briefly, the bacteria tested were first inoculated in TSBS, after two days, a 10 μL drop was transferred to the centre of a plate with tryptic soy agar supplemented 2% NaCl (TSAS), which was then incubated for 2–3 days at 20 °C. When colonies appeared, the dishes were exposed to chloroform vapour for 20 min, and a second

layer of semisolid TSAS (8 g agar l^{-1} of tryptic soy broth supplemented with 2% NaCl), in which the pathogenic strain had been previously inoculated, was poured on each plate. Each test was run in duplicate and inhibition was considered positive only if a clear zone area was apparent in both duplicates.

Metanauplii of *Artemia franciscana*, hatched from decapsulated cysts (Sorgeloos et al., 1977) and enriched 18 h with 300 ppm Rich Advance (Rich, Greece), were used to determine the effect of incubation in microalgae on the microbial load of enriched *A. franciscana* metanauplii. *A. franciscana* metanauplii were incubated in cultures of *T. Chuii* or, *C. minutissima*, or seawater (control for 30 min at room temperature (20–22 °C). The density of microalgae used in this experiment was 2×10^6 and 5×10^7 cells ml^{-1} for *T. chuii* and *C. minutissima*, respectively. The metanauplii were then stored at −20 °C for two days. Approximately 2 g of thawed metanauplii were transferred to Petri dishes with filtered seawater for 1 h. Microbiological samples were taken from live *Artemia* metanauplii after incubation in microalgae (or seawater), and from thawed *Artemia* metanauplii incubated in filtered seawater after one h. In each case, 10 metanauplii were homogenized in 2 ml of sterile 80% seawater and 10-fold dilutions of this homogenates were plated on agar dishes with Zobell's marine agar and TCBS medium. The dishes were stored at 20 °C, and the numbers of CFU were counted daily for 7–10 days.

One way ANOVA was used to compare data, and if the test showed significant *F* values, Tukey's test was used for the multiple comparisons. Statistical analyses were run using the software program STATGRAPHICS Plus 5.0.

3. Results

High numbers of CFU were detected in supernatants from cultures of both microalgae species used (Fig. 1). The numbers of bacteria associated with the microalgae cultures showed an exponential growth during the experiment and they were higher in *T. chuii* in all three sampling points compared with *C. minutissima*. No growth of bacteria was observed, however, in samples plated on TCBS medium.

A total of 17 and 30 bacterial strains were isolated from *C. minutissima*, and *T. chuii*, respectively. A high percentage of Gram-positive strains were detected among these isolates (Table 1), which constituted 82% (14/17), and 73% (22/30) of the total numbers of isolates in *C. minutissima* and *T. chuii*, respectively. All strains isolated at the first sampling point (2 days after inoculation) from both microalgae species were Gram-positive.

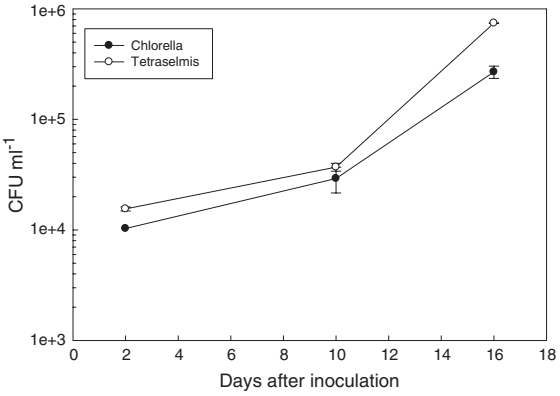

Fig. 1. Numbers of CFU ml^{-1} (±SE, $n=2$) in supernatant from cultures of *C. minutissima* and *T. chuii* after 2, 10, and 16 days after inoculation.

Nine of the 34 strains tested (31%) showed inhibition in vitro against either of the two pathogenic strains. Four of these strains were isolated from *C. minutissima* and five from *T. chuii* cultures. Of those nine bacterial strains that showed inhibition, five strains were Gram-positive and four were Gram-negative (Table 1).

The numbers of CFU in homogenates of *Artemia* incubated in microalgae cultures were significantly lower ($P<0.05$) than those of CFU in homogenates of *Artemia* from the control treatment (Fig. 2). Similarly, the numbers of CFU in *Artemia* homogenates after the simulation experiment were significantly lower ($P<0.05$) in the treatments incubated in the microalgae compared with seawater. The differences in CFU in all cases between the two treatments with microalgae were not significant ($P>0.05$). The numbers of CFU in TCBS were higher in the control group, compared with the treatments incubated in microalgae cultures. The percentage of presumptive *Vibrio*, as calculated from the numbers of CFU in *Artemia* homogenates plated on TCBS and TSBS in *C. minutissima*, *T. chuii* and seawater, were 0%, 32% and 40%, respectively.

4. Discussion

The numbers of CFU ml^{-1} in the microalgae supernatants indicated that there was an increased proliferation of bacteria in the microalgae cultures during the stationary phase (10 and 16 days after inoculation) in comparison with the exponential phase (2 days after inoculation). Similar results have been obtained with batch cultures of six different microalgae

Table 1
Basic biochemical tests for phenotypic characterization of the bacterial strains isolated and inhibition against *P. damselae* (P) and *V. anguillarum* (V)

Isolate	Source	*d*	Gram	Oxidase	Catalase	Na^+	O/F	TCBS	A	L	O	Amylase	Inhibition
3T1	Chlo	2	+	–	+	+	I	–	+	+	+	–	nd
3T2	Chlo	2	+	+	+	+	I	–	+	+	+	nd	None
3T3	Chlo	2	+	+	+	+	I	–	+	+	+	–	nd
3T4	Tetra	2	+	–	+	+	I	–	+	+	+	–	None
3T5	Tetra	2	+	–	+	+	F	w	+	+	+	w	None
3T6	Tetra	2	+	–	–	+	I	–	+	+	+	–	V
3T7	Tetra	2	+	w	–	+	I	–	+	+	+	–	None
3T8	Tetra	2	+	+	–	+	I	–	+	+	+	–	P
3T9	Tetra	2	+	+	nd	+	I	–	+	+	+	–	None
3T10	Tetra	2	+	+	+	+	I	w	+	+	+	–	None
3T11	Tetra	2	+	+	+	+	I	–	+	+	+	–	None
3T12	Tetra	2	+	+	+	+	I	–	+	+	W	–	None
3T13	Tetra	2	+	–		+	I	–	nd	nd	nd	–	nd
3T14	Tetra	2	+	+	+	+	I	–	+	+	+	–	None
3T15	Chlo	10	+	–	+	+	I	–	+	+	+	–	nd
3T16	Chlo	10	+	–	+	+	I	–	+	+	+	–	nd
3T17	Chlo	10	+	–	+	+	nd	–	+	+	+	–	None
3T18	Chlo	10	+	–	–	+	nd	nd	nd	nd	nd	nd	nd
3T19	Chlo	10	+	+	+	+	I	–	+	+	+	–	P
3T20	Tetra	10	+	–	+	+	O/F	–	+	+	+	–	None
3T21	Tetra	10	+	–	+	+	F	–	+	+	+	–	nd
3T22	Tetra	10	+	–	+	+	F	–	+	+	+	–	None
3T23	Tetra	10	+	+	+	+	I	–	+	+	+	–	None
3T24	Tetra	10	–	+	+	–	F/O	–	+	+	+	–	V
3T25	Tetra	10	–	w	+	–	F	–	+	+	+	+	P
3T26	Tetra	10	–	+	+	–	I	–	+	+	+	+	None
3T27	Tetra	10	–	w	+	–	I	–	+	+	+	+	nd
3T28	Tetra	10	–	+	+	–	I	–	+	+	+	–	None
3T29	Chlo	10	+	–	w	+	I	–	+	+	–	–	None
3T31	Chlo	10	–	+	+	–	I	–	+	+	+	–	None
3T33	Chlo	10	–	+	+	–	I	–	+	+	+	–	V
3T34	Tetra	16	+	+	+	+	F	w	+	+	–	+	nd
3T35	Tetra	16	–	+	+	–	I	–	+	+	+	+	V
3T36	Tetra	16	+	+	+	+	F	–	+	+	+	–	nd
3T39	Tetra	16	–	+	+	–	I	–	+	+	+	–	None
3T40	Tetra	16	+	+	+	+	I	–	+	+	+	–	None
3T41	Chlo	16	+	+	+	+	F	–	+	+	+	–	V
3T42	Chlo	16	+	+	+	+	I	–	+	+	+	–	None
3T43	Chlo	16	+	+	+	+	I	–	+	+	+	–	nd
3T44	Tetra	16	+	+	+	+	F	–	+	+	+	–	None
3T45	Tetra	16	+	nd	–	+	I	–	+	+	+	–	None
3T46	Tetra	16	–	w	+	–	I	–	+	+	+	+	None
3T47	Tetra	16	+	–	+	+	I	–	+	+	–	–	nd
3T49	Chlo	16	+	w	+	+	I	–	+	+	+	–	nd
3T50	Chlo	16	–	+	+	–	I	–	+	+	+		None
3T51	Chlo	16	+	+	+	+	F	–	+	+	+		V, P
3T52	Tetra	16	+	+	nd	+	nd	–	+	+	+		None

Chlo: *Chlorella minutissima*, Tetra: *Tetraselmis chuii*, I: inert, F: fermentation, O: Oxidation, w: weak reaction, plus sign in Na^+ column indicates that a bacterial strain grew in the absence of Na^+ ions, whereas plus sign indicates oxidase, amylase and catalase activity in the respective columns; A, L, and O indicate arginine, leucine and ornithine decarboxylase, nd: not determined.

species by Salvesen et al. (2000), where higher concentrations of bacteria were associated with microalgae cultures in the stationary phase compared with the exponential phase. Despite the large increase in the bacterial load of the cultures, no growth of presumptive *Vibrio* was observed in TCBS agar, although members of this group are ubiquitous in the marine environment (Rheinheimer, 1977). The lack of presumptive *Vibrio*

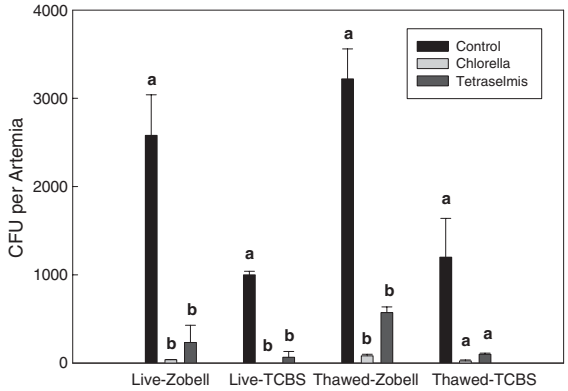

Fig. 2. Numbers of CFU per *Artemia* in homogenates of live and thawed *Artemia* plated in Zobell's marine agar and TCBS medium. Columns assigned unlike letters at each sampling indicate differences between the three treatments.

among the dominant strains indicated that some factors might have inhibited their growth in the microalgae cultures. Such inhibitory mechanisms may include bactericidal action or competition of other bacterial populations associated with the microalgae or production of antimicrobial substances by the microalgae cells. Antimicrobial activity has previously been demonstrated in cultures of *Tetraselmis suesica* (Austin et al., 1992) and *C. minutissima* (Katharios et al., 2005), although the mechanisms of action of this antimicrobial activity have not been determined.

Many Gram-positive bacterial strains were detected in the cultures of the two microalgae species. This could mean that any possible antimicrobial activity might have been selectively directed against Gram-negative bacterial strains only. A significant proportion (9/34) of the strains tested from the bacterial strains isolated from cultures of the two microalgae species showed inhibition in vitro against bacterial pathogens of fish. Results from in vitro experiments cannot, however, be extrapolated to explain the microbial condition found in the microalgae cultures. It has been shown that such inhibition in vitro of pathogens does not necessarily correspond to any significant effect in routine culture conditions (Gram et al., 2001).

The effect of the short-term incubation of *Artemia* metanauplii in microalgae cultures on the nutritional value of metanauplii was not determined. Nevertheless, since the gut of the *Artemia* metanauplii is emptied of their content in oil-emulsion and filled with algae, the HUFA content of *Artemia* had probably decreased after the incubation in microalgae.

The results of this study demonstrated a simple approach to decrease the microbial load and at the same time reduce the percentage of *Vibrio* among the bacteria associated with enriched *Artemia*. This is of practical importance for marine fish hatcheries because of its simplicity and, in most cases, because marine hatcheries have access to microalgae such as *T. chuii* and *C. minutissima*. Addition of probiotic in live food cultures could be an alternative, but it requires addition of probiotics during the enrichment process in very high numbers of bacteria (10^8–10^{10} cells ml^{-1}) to have a significant effect on the bacterial load of the Artemia (Gatesoupe, 2002; Vilamil et al., 2003). This high demand in live bacterial cells makes this approach difficult to implement in practice. Similar results to our study have been observed in juvenile *Artemia* incubated in microalgae for 4 h (Olsen et al., 2000). It was shown here, however, that a much shorter period is sufficient to reduce the bacterial load in *Artemia* metanauplii. Several species of microalgae of six different classes produced superoxide (Marshall et al., 2005), so the production of superoxide might explain the bactericidal effect of the microalgae in cultures of *Artemia* shown in our study.

Acknowledgements

This work was supported by the research project "Probiotics and immune modulation in larvae and juveniles of marine fish", ref. nr. 38781/BSE/2001 funded by Fundação para a Ciência e a Tecnologia, Portugal. We thank the Spanish Type Culture Collection for supplying us with the bacterial strains, Silvia Martins for her technical assistance in with microbiology work, and Dr Peter C. Hubbard for reviewing the manuscript.

References

Austin, B., Allen, D.A., 1982. Microbiology in laboratory-hatched brine shrimp (*Artemia*). Aquaculture 26, 369–383.

Austin, B., Baudet, E., Stobie, M., 1992. Inhibition of bacterial fish pathogens by *Tetraselmis suesica*. J. Fish Dis. 15, 55–61.

Dopazo, C.P., Lemos, M.L., Lodeiros, C., Bolinches, J., Barja, J.L., Toranzo, A.E., 1988. Inhibitory activity of antibiotic-producing marine bacteria against fish pathogens. J. Appl. Bacteriol. 65, 97–101.

Duff, D.C.B., Bruce, D.L., 1966. The antimicrobial activity in marine planktonic algae. Can. J. Microbiol. 12, 877–884.

Gatesoupe, F.J., 2002. Probiotic and formaldehyde treatment of *Artemia* nauplii as food for larval Pollack, *Pollachius pollachius*. Aquaculture 212, 347–360.

Gram, L., Løvold, T., Nielsen, J., Melchiorsen, J., Spanggaard, B., 2001. In vitro antagonism of the probiont *Pseudomonas fluorescens* strain AH2 against *Aeromonas salmonicida* does not confer protection of salmon against furunculosis. Aquaculture 199, 1–11.

Guillard, R.R.L., Ryther, J.H., 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hudstedt, and *Detonula converfacea* (Cleve) Gran. Can. J. Microbiol. 8, 229–239.

Imsland, A.K., Foss, A., Conceição, L.E.C., Dinis, M.T., Delbare, D., Schram, E., Kamstra, A., Rema, P., White, P., 2003. A review of the culture potential of *Solea solea* and *S. senegalensis*. Rev. Fish Biol. Fish. 13, 379–407.

Katharios, P., Papadakis, I.E., Prapas, A., Dermon, A.C., Ampatzis, K., Divanach, P., 2005. Mortality control of viral encephalopathy and retinopathy in 0+ grouper *Epinephelus marginatus* after prolonged bath in dense *Chlorella minutissima* culture. Bull. Eur. Assoc. Fish Pathol. 25, 28–31.

Makridis, P., Fjellheim, J.A., Skjermo, J., Vadstein, O., 2000a. Colonization of the gut in first feeding turbot by bacterial strains added to the water or bioencapsulated in rotifers. Aquac. Int. 8, 367–380.

Makridis, P., Fjellheim, J.A., Skjermo, J., Vadstein, O., 2000b. Control of the bacterial flora of *Brachionus plicatilis* and *Artemia franciscana* by incubation in bacterial suspensions. Aquaculture 185, 207–218.

Marshall, J.A., de Salas, M., Oda, T., Hallegraeff, G., 2005. Superoxide production by marine microalgae—I. Survey of 37 species from 6 classes. Mar. Biol. 147, 533–540.

Oie, G., Reitan, K.I., Olsen, Y., 1994. Comparison of rotifer culture quality with yeast-plus oil and algal based diet cultivation diets. Aquac. Int. 2, 225–238.

Oie, G., Makridis, P., Reitan, K.I., Olsen, Y., 1997. Protein and carbon utilization of rotifers (*Brachionus plicatilis*) in first feeding of turbot larvae (*Scophthalmus maximus* L.). Aquaculture 153, 103–122.

Olafsen, J.A., 2001. Interactions between fish larvae and bacteria in marine aquaculture. Aquaculture 200, 223–247.

Olsen, A.I., Olsen, Y., Attramadal, Y., Christie, K., Birkbeck, T.H., Skjermo, J., Vadstein, O., 2000. Effects of short term feeding of microalgae on the bacterial flora associated with juvenile *Artemia franciscana*. Aquaculture 190, 11–25.

Reitan, K.I., Rainuzzo, J.R., Oie, G., Olsen, Y., 1997. A review on the nutritional effects of algae in marine fish larvae. Aquaculture 155, 207–221.

Rheinheimer, G., 1977. Regional and seasonal distribution of saprophytic and coliform bacteria. In: Rheinheimer, G. (Ed.), Microbial Ecology of a Brackish Water Environment. Ecological Studies, vol. 25. Springer-Verlag, Berlin, pp. 121–137.

Ritar, A.J., Dunstan, G.A., Nelson, M.M., Brown, M.R., Nichols, P.D., Thomas, G.W., Smith, E.G., Crear, B.J., Kolkovski, S., 2004. Nutritional and bacterial profiles of juvenile *Artemia* fed different enrichments and during starvation. Aquaculture 239, 351–373.

Salvesen, I., Skjermo, J., Vadstein, O., 1999. Growth of turbot (*Scophthalmus maximus* L.) during first feeding in relation to the proportion of r/K strategists in the bacterial community of the rearing water. Aquaculture 175, 337–350.

Salvesen, I., Reitan, K.I., Skjermo, J., Øie, G., 2000. Microbial environments in marine larviculture: impacts of algal growth on the bacterial load in six microalgae. Aquac. Int. 8, 275–287.

Smibert, R.M., Krieg, N.R., 1994. Phenotypic characterization. In: Gerhardt, P., Wood, W.A., Krieg, N.R. (Eds.), Methods for General and Molecular Biotechnology. American Society for Microbiology, Washington, D.C., USA, pp. 607–654.

Sorgeloos, P., Bossuyt, E., Laviña, E., Baeza-Mesa, M., Persoone, G., 1977. Decapsulation of *Artemia* cysts: a simple technique for the improvement of the use of brine shrimp in aquaculture. Aquaculture 12, 311–315.

Vilamil, L., Figueras, A., Planas, M., Novoa, B., 2003. Control of *Vibrio alginolyticus* in *Artemia* culture by treatment with bacterial probiotics. Aquaculture 219, 43–56.

Zobell, C.E., 1946. Marine Microbiology: A Monograph on Hydrobacteriology. Cronica Botanica. Waltham, MA, USA.